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PRINCIPAL INVESTIGATOR: Dr. David C. Ward

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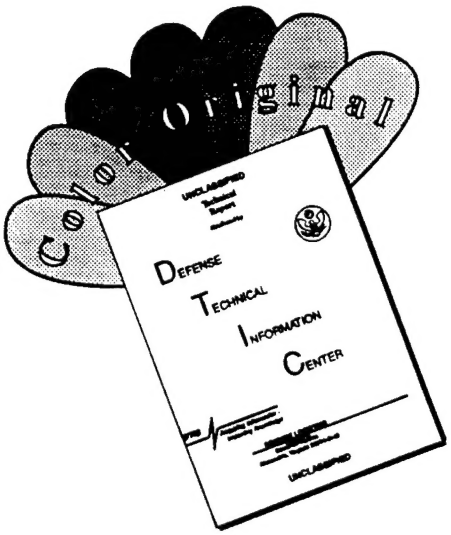

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The overall objective of this grant is to develop new multiparametric fluorescence *in situ* hybridization (M-FISH) techniques for the analyses of chromosomal abnormalities and specific genes implicated in the initiation and progression of breast carcinomas. During year 01, we identified seven distinct fluorophores and developed optical filter sets to discriminate each of these fluorophores. By using different combinations of fluorophores for probe labeling, we have been able to hybridize up to 27 probes similtaneously, each being identified by its unique spectral signature. We have developed a novel method of chromosome karyotyping using M-FISH such that each chromosome is "painted" a different color. This technique is being used to karyotype chromosome preparations from short-term cultures of breast tumor tissue obtained by needle aspiration. Comparative genome hybridization (CGH) is also being used to define genetic changes that occur in breast tumor cells at various stages of malignancy. Extension of M-FISH to fresh, frozen and paraffin-embedded tissues is in progress. Data from these studies will be assessed with other histological, molecular, and immunological information and correlated with tumor site, size, lymph node status, metatasis, therapy and survival, once a statistically significant number of samples have been analyzed.

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David C Hall 10/25/95

PI - Signature Date

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Introduction

Breast cancer is a heterogeneous disease with complex genetic changes. Knowledge of the genetic changes occurring during the initiation and progression of breast neoplasia is necessary for the development of new diagnostic markers, the identification of potential new therapeutic modalities, the rational analyses of treatment protocols, improving the prognosis of remission or metastatic potential and assessing genetic risk status.

The overall objective of this project is to develop new fluorescence *in situ* hybridization (FISH) techniques that would permit the analysis of multiple genetic loci simultaneously in breast tumor tissues or on chromosomes prepared from short term cultures of breast tumor cells. A large number of genes (or specific chromosomal regions) have already been implicated in the etiology of breast carcinoma. Many of these genetic loci have been shown to be deleted or amplified in tumor cells using molecular techniques, such as loss of heterozygosity (LOH) or comparative genome hybridization (CGH). Generally, these methods provide information on the tumor as a whole since they utilize DNA extracted from large numbers of tumor cells. The ability to assess the status of multiple genetic loci simultaneously *in situ* in fresh, frozen or paraffin-embedded tissues could not only provide a rapid means for detecting the loss or amplication of these loci, but it would do so on a cell by cell basis that can be related directly to tissue histology. The detection of chromosomal abnormalities at the metaphase chromosome level has also proven problematic because of the difficulty in preparing high quality metaphase spreads from cultured breast tissue epithelial cells and the complexity of the chromosomal rearrangements that are generally found. However, recent advances in tissue culture technology has made it possible to obtain chromosome preparations with high efficiency from short term culture of breast tumor cells obtained by needle aspiration. This provides an opportunity to obtain karyotype data on breast tumors which can be incorporated into the total data set (histological, pathologic, molecular and genetic) used in the diagnosis and treatment of patients.

To simplify and automate cancer karyotyping and the multiplex analysis of the numerous genes implicated in the etiology of breast neoplasias, we have set out to extend the analytic capabilities of fluorescence *in situ* hybridization (FISH) techniques. This report presents the progress we have made during the first year of grant support toward developing an automated color karyotyping microscope system and a multiplex FISH method for monitoring gene amplication and loss of heterozygosity directly in tissue sections.

Progress Report

All of our effort during year 01 were focused on the identification of appropriate sets of spectrally resolvable fluorophores and optical filter sets that would permit the analysis of 6 to 7 fluors simultaneously and to utilize chromosomal DNA "painting" libraries, combinatorially labeled with these fluors, to carry out 24 color FISH karyotyping. The results obtained to date are outlined below.

Results

Selection of resolvable fluors and high contrast filters.

The limited spectral bandwidth available for fluorescence imaging (~350-800 nm), and the extensive overlap between the spectra of organic fluors, makes separating multiple fluors spectroscopically a significant technical challenge. Choice of the fluors and filters in Table 1 (See Appendix with Figures and Tables) was based on computer modeling of their contrast parameters using digitized excitation and emission spectra of a number of common fluors, the transmittance spectra of interference filters and dichroic beamsplitters from several optical supply houses (Omega, Ealing, Oriel, Zeiss), and the power spectra of the high pressure mercury

arc, high pressure xenon arc, the Hg-Xe arc, the quartz tungsten-halogen lamp and various laser excitation sources. Excitation and emission contrast ratios were computed for every fluor relative to its two nearest spectral neighbors. Filters were then selected to achieve >90% discrimination. This level of contrast is not generally achievable using either excitation selection or emission selection alone, no matter how narrow the filter bandwidths. Thus excitation selection and emission selection were applied simultaneously. Excitation contrast was favored whenever possible, to avoid unnecessary wasting of fluorescence photons (and hence unproductive photobleaching of the fluor).

The chosen fluor set consisted of 4'-6-diamidino-2-phenylindole (DAPI, a general DNA counterstain), fluorescein (FITC), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. The absorption and emission maxima, respectively, for these fluors are: DAPI (350nm; 456nm), FITC (490nm; 520nm), Cy3 (554nm; 568nm), Cy3.5 (581nm; 588nm), Cy5 (652nm; 672nm), Cy5.5 (682nm; 703nm) and Cy7 (755nm; 778nm). The excitation and emission spectra, extinction coefficients and quantum yield of these fluors are described in detail elsewhere. All fluors were excited with a 75W Xenon arc .

To attain the required selectivity, filters with bandwidths in the range of 5-15 nm (cf. approximately 50 nm or more for "standard" filter sets) were required. Further, it was necessary to both excite and detect the fluors at wavelengths far from their spectral maxima. Emission bandwidths were made as wide as possible. For low-noise detectors such as the cooled CCD camera used here, restricting the excitation bandwidth has little effect on attainable signal to noise ratios; the only drawback is increased exposure time.

Epifluorescence filter cubes constructed using the bandpass and dichroic filters listed in Table 1 were tested experimentally for spectral contrast between adjacent fluors. Good contrast (>92%) was attained in practice for all fluors except the Cy5/Cy 5.5 pair, which was marginal (80-90%). For this reason, Cy 5.5 was omitted from the combinatorial labeling experiments described below. It is vital to prevent infra-red light emitted by the arc lamp from reaching the detector; CCD chips are extremely sensitive in this region. Thus, appropriate IR blocking filters (Table 1) were inserted in the image path immediately in front of the CCD window, to minimize loss of image quality.

Software for analysis of combinatorially labeled probes.

Multi-fluor combinatorial labeling depends on acquiring and analyzing the spectral signature of each probe, i.e. the intensity values of each of the component fluors in the six imager channels. Critical features are accurate alignment of source images, correction of chromatic crosstalk, and quantitation of the intensity of each fluor. To circumvent manual image manipulation we developed the necessary software to achieve these functions. This software carries out the following steps in sequential order:

1) correction of geometric image displacements caused by wedging in the emission interference filters, and by mechanical noise, 2) calculation of a DAPI segmentation mask to delineate all chromosomes in a metaphase spread, 3) calculation and subtraction of background intensity values for each fluor, calculation of a threshold value, and creation of a segmentation mask for each fluor. 4) use of the segmentation mask of each fluor to establish a "boolean" spectral signature of each probe, 5) display the material hybridizing to each chromosome probe next to the DAPI image to facilitate chromosome identification 6) create a composite gray value image, where the material from each chromosome is encoded with a gray value and 7) final presentation of the hybridization results using a look-up table (LUT) that assigns a pseudocolor to each such gray value.

Color karyotyping of normal human chromosomes.

To test the feasibility of karyotyping chromosomes by M-FISH, chromosome painting probes representing the 22 autosomes and two sex chromosomes were combinatorially labeled, mixed, and cohybridized to normal metaphase spreads prepared from peripheral blood lymphocytes. In the case of chromosomes 3, 5 and 11 separate p and q arm probes were used; thus a total of 27 different fluor combinations were tested in this experiment. The DNA probes were generated by microdissection and subsequent PCR amplification and labeled by nick translation. As expected, probes labeled with equal amounts of different fluors did not give equal signal intensities for each fluor, reflecting the fact that the filter sets were selected to maximize spectral resolution rather than throughput. To diminish signal intensity differentials, probe concentrations for the hybridization mix had to be established carefully in a large number of control experiments.

Figure 1 shows a metaphase spread after hybridization with the 27 probe cocktail. The fluorescence source images for DAPI, FITC, Cy3, Cy3.5, Cy5 and Cy7 are shown in the left column of the figure while the segmentation masks computed for each fluor are shown in the right column. After subtracting the interchromosomal fluorescence background from the chromosomal fluorescence, the mean of the intrachromosomal fluorescence intensities was used to calculate the threshold for creating the individual segmentation mask of each fluor. Discrimination of hybridization positive and hybridization negative chromosomes was not problematic and was accomplished within a second or two. Figure 2 shows a typical distribution of the relative fluorescence intensity values for a single fluor, Cy3.5, that documents the signal differential between hybridization positive and hybridization negative pairs of homologous chromosomes; all chromosomes showing fluorescence intensities above the threshold are represented in the Cy3.5 mask.

Table II lists the fluors used to generate the spectral signatures for each chromosome. Each of the fluor segmentation masks is interrogated by the computer program in order to establish the fluor composition of all chromosomes. For example, chromosome 7 material should be labeled with FITC, Cy3 and Cy3.5 while chromosome 9 material is labeled with FITC, Cy3 and Cy5. Each of the individual chromosomes (or chromosomal segments) identified in the fluor masks on the basis of their fluor composition (i.e. its boolean spectral signature) can be displayed on the computer monitor next to the DAPI image of the metaphase spread. This provides a simple means for the operator to confirm chromosome identity and to assess any chromosomal abnormalities seen in the final gray value or pseudocolored composite images. Figure 3 documents the identity of two of the chromosome pairs, chromosome 7 and chromosome 9, present in the metaphase spread shown in Figure 1.

Figure 4a shows the metaphase spread in Figure 1 as a pseudocolored image while Figure 4b illustrates the karyotype generated on the basis of the spectral signature of each chromosome. Note that the p and q of chromosome 3, 5 and 11 are labeled differentially, reflecting the use of the arm specific probes. The karyotype is that expected for a normal male cell. Multiple chromosome spreads were analyzed in a similar fashion and all indicated a normal karyotype as expected. The high efficiency of the chromosome painting achieved and the reproducibility of these results through many additional experiments using either 24 or 27 color labeling protocols indicated that karyotype analysis by M-FISH was indeed feasible.

Analysis of chromosomal abnormalities by M-FISH.

To further test the utility of M-FISH for karyotype analysis, we examined a set of five patient samples in which various chromosomal alterations had been detected by conventional cytogenetic banding techniques. These samples were provided by the Yale University Clinical Cytogenetics Laboratory and were analyzed in a blind fashion using a pool of 24 whole chromosome painting probes. Figure 5 shows the karyotypes

determined by M-FISH for each of the five patient specimens. The chromosomal abnormalities observed were as follows: BM2486, a 5;8 translocation (Fig. 5a); 10608, a 2;14 translocation (Fig. 5b); BM2645, a 2;3 translocation plus a deletion in 6q (Fig. 5c); BM2527, a 15;17 translocation (Fig. 5d), and BM3149, a 3;5 and a 6;12 translocation, loss of one copy of chromosomes 5 and 12 and trisomy of chromosome 8 (Fig. 5e). In each case, the chromosomal alterations seen by M-FISH were identical to those identified by cytogenetic banding. Furthermore, using the G-banding pattern generated by the DAPI fluorescence and measuring the fractional chromosome length of the translocation chromosomes, it was possible to infer the cytogenetic band implicated in the translocation breakpoints using fractional chromosome length/cytogenetic band conversion charts which again agreed with the cytogenetic data. In some instances, e.g. BM2486, BM2645 and BM2527, where the chromosomes are highly condensed there is an additional color generated at the site of the translocation breakpoints. This is due the blending of colors by fluorescence flaring at the junctions of the individual chromosome painting probe domains. This color blending is not observed when more extended, e.g. prometaphase chromosomes, are examined (e.g. sample 10608), or when the fluor composition of both translocation chromosomes have only a single fluor difference. For example in BM2527 (Fig. 5 d) chromosome 15 was labeled with FITC, Cy3.5 and Cy7, while chromosome 17 was labeled with FITC and Cy7 only; in this translocation no color blending is observed. Color blending also occurs at sites where two different chromosomes overlap in the spread. However, by examining several spreads potential problems in chromosome characterization can be avoided.

Previous studies have shown that G or R banding profiles can be generated by hybridization with oligonucleotides complementary to LINE or SINE (Alu) sequences. Although such banding probes were not used in these experiments, it is clearly feasible to do so. However, since it is preferable to use a fluor that does not contribute to the spectral signature of the chromosomal DNAs to label the banding probe, we chose not to include hybridization banding in our protocol until we could refine the spectral resolution between Cy5 and Cy5.5. This would provide the additional fluor, Cy5.5, needed for such experiments.

We next studied a pair of cell lines HTB43 and SCC15, derived from patients with squamous cell carcinoma of the head and neck. These cell lines possessed sufficiently extensive chromosomal rearrangements that much of their chromosome complement were designated as "marker" chromosomes when analyzed by a board certified cytotechnologist using conventional cytogenetic banding procedures. The G-banded karyotype of one HTB43 cell is shown in Figure 6a. Information gleaned by inspection of the M-FISH images was used to assemble the DAPI banded karyotype of SCC15 (Figure 7a) since chromosome identification based on either DAPI or G-banding patterns alone also left a large number of chromosomes unidentified. The karyotypes of these cell lines as revealed by M-FISH are illustrated in Figures 6b and 7b; in these experiments we again used arm specific probes for chromosomes 3, 5 and 11. The chromosomal partners in numerous chromosomal translocations are readily identifiable and some of the chromosomal rearrangements are extremely complex. Many but not all metaphase spreads for SCC15 demonstrated some double minute chromosomes (DMs). In the metaphase spreads shown in Figs. 7a and b one single DM was observed and identified as chromosome 1 material. This demonstrates the usefulness of M-FISH to identify DMs. A schematic representation of all the chromosomal rearrangements observed in HTB43 and SCC15 are shown in Fig. 6c and Fig. 7c, respectively.

Comparative genome hybridization (CGH) was also done on cell lines HTB43 and SCC15 to determine those chromosomal regions that were over- or underrepresented in the tumor lines (data not shown). Both cell lines have a large number of different clones. Since CGH provides a summary of data for all clones one

would not necessarily expect that the M-FISH data from a small number of metaphase spreads would correlate exactly with these relative copy number karyotypes. However, even with this caveat, there was a very close similarity between the M-FISH and CGH data concerning the relative abundance of whole chromosomes or chromosome segments. Since the CGH data indicated that chromosome 3p was underrepresented in these cell lines while 3q, 5p, and 11q arms were overrepresented, the use of the arm specific probes for these chromosomes in M-FISH facilitated the comparative evaluation.

M-FISH with band-specific probes and YAC clones

A set of three band specific probes from chromosome 6 were used with 19 non chimeric YAC clones and two whole chromosome painting probes (for chromosomes X and 8) in order to determine if M-FISH could be done efficiently using regionally localized and genetically less complex probe mixtures. The result of this experiment, shown in Fig. 8, demonstrates that both band specific clone pools and individual YAC clones with inserts as small as 0.5 kb can be imaged and their spectral signatures correctly identified by our computer algorithms. While the ultimate sensitivity of M-FISH has yet to be established, it should be possible to assemble probe panels to address a broad spectrum of specific biological and clinical questions.

Appendix

Tables and Figures

Table 1: Epicube Filter Configuration for 75 W Xe Arc Source

	DAPI	FITC	Cy3	Cy3.5	Cy5	Cy7
Excitation Filter	Zeiss 365 nm	Omega 455DF70	Omega 546DF10	Ealing 35-3763	Omega 640DF20	Omega 740DF25
Dichroic Beamsplitter	Zeiss 395 nm	Omega 505DRLP02	Omega 560DRLP02	Omega 590DRLP02	Omega 645DRLP02	Omega 777DRLP02
Emission Filter	Zeiss > 397 nm	Omega 530DF30	Ealing 35-3722	Zeiss 630/30	Omega 670DF32	Omega 780EFLP
IR Blocking	Schott BG38	Schott BG38	Schott BG38	Schott BG38	Oriel 58893	Oriel 58895

Table 2: Combinatorial labeling scheme used for the simultaneous labelling of all 22 autosomes and the two sex chromosomes.

a) 24 colors

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Fluoroscein	x					x	x		x				x		x		x			x	x	x		x
Cy3			x		x		x		x			x	x			x			x				x	x
Cy3.5				x		x	x				x	x		x	x			x	x		x		x	
Cy5								x	x	x	x			x		x			x	x	x	x		
Cy7		x			x			x				x	x	x	x		x	x		x				

b) 27 colors

	1	2	3p	3q	4	5p	5q	6	7	8	9	10	11p	11q	12	13	14	15	16	17	18	19	20	21	22	X	Y
Fluoroscein	x							x	x		x		x			x		x		x			x	x	x		x
Cy3			x	x		x	x		x		x				x	x			x			x				x	x
Cy3.5					x	x		x	x				x	x	x		x	x			x	x		x		x	
Cy5			x			x				x	x	x	x	x			x		x			x	x	x	x		
Cy7		x	x			x	x			x			x		x	x	x	x		x	x		x				

Figure Legends

Fig. 1

Normal male metaphase spread after hybridization with a 27 DNA-probe cocktail. The left column of the figure shows the unprocessed fluorescence source images and the right column the segmentation masks computed for each fluor.

a) DAPI: The DAPI source image was inverted in order to produce a G-band like pattern; b) FITC; c) Cy3; d) Cy3.5; e) Cy5; f) Cy7

Fig. 2

Plot of the relative fluorescence intensity values for the fluor Cy3.5. Each pair of dots represents the mean fluorescence intensity values of the respective chromosome homologues. Note that chromosomes that were labeled with fluors very close to Cy3.5 in the spectrum, i.e. Cy3 or Cy5 but not with Cy3.5 do not have significantly increased intensity values. For example, chromosome 16 was labeled with Cy3 and Cy5 only. The intensity values over these chromosomes were no higher than any other chromosome not labeled with Cy3.5. This demonstrates the specificity of the filter used, thus allowing the unequivocal discrimination of hybridization positive and negative chromosomes.

Fig. 3

Display of individual chromosome pairs identified on the basis of their fluor composition next to the DAPI image of the metaphase spread. This part of the computer program facilitates the identification of chromosome material in the metaphase spread. Identification of a) chromosome 7 and b) chromosome 9.

Fig. 4

a) Metaphase spread of Fig. 1 as a pseudocolored image. For chromosomes 3, 5 and 11 the p and q arms were labeled differently.

b) Final karyotype generated on the basis of the boolean spectral signature.

Fig. 5

Examples of chromosomal abnormalities detected by 24 color M-FISH.

a) BM2486: 46, XX, t(5;8)

b) 10608: 46, XY, t(2;14)

c) BM2645: 46, XX, t(2;3), del 6(q) (arrow)

d) BM2527: 46, XY, t(15;17)

e) BM3149: 45, XY, -3, +der[t(3;5)], -5, -6, +der[t(6;12)], +8, -12

Samples a and b were from individuals with constitutional translocations.

Samples c, d, e were from patients with acute myeloid leukemia.

Fig. 6

a) G-banded karyotype of the cell line HTB43. The cell shown here has 57 chromosomes. Many of the chromosomal rearrangements could not be deciphered with conventional cytogenetic banding procedures.

b) M-FISH analyses of the cell line HTB43. This cell has 49 chromosomes and some identical marker chromosomes as the cell shown in a). Two marker chromosomes contain chromosome material in a band like fashion. One has a p-arm consisting of 5p, while the q-arm has one band consisting of chromosome 14 material and then two alternating bands with chromosome 9 and 11q material. The second marker chromosome is a 15p+ with alternating bands of 3q and chromosome 8 material.

c) A schematic representation of the chromosomal rearrangements observed in the metaphase shown in b).

Fig. 7

a) DAPI banded karyotype of a metaphase spread from cell line SCC15. The chromosomes were arranged based on the M-FISH results shown in b).

b) M-FISH analyses of the same metaphase spread as shown in a).

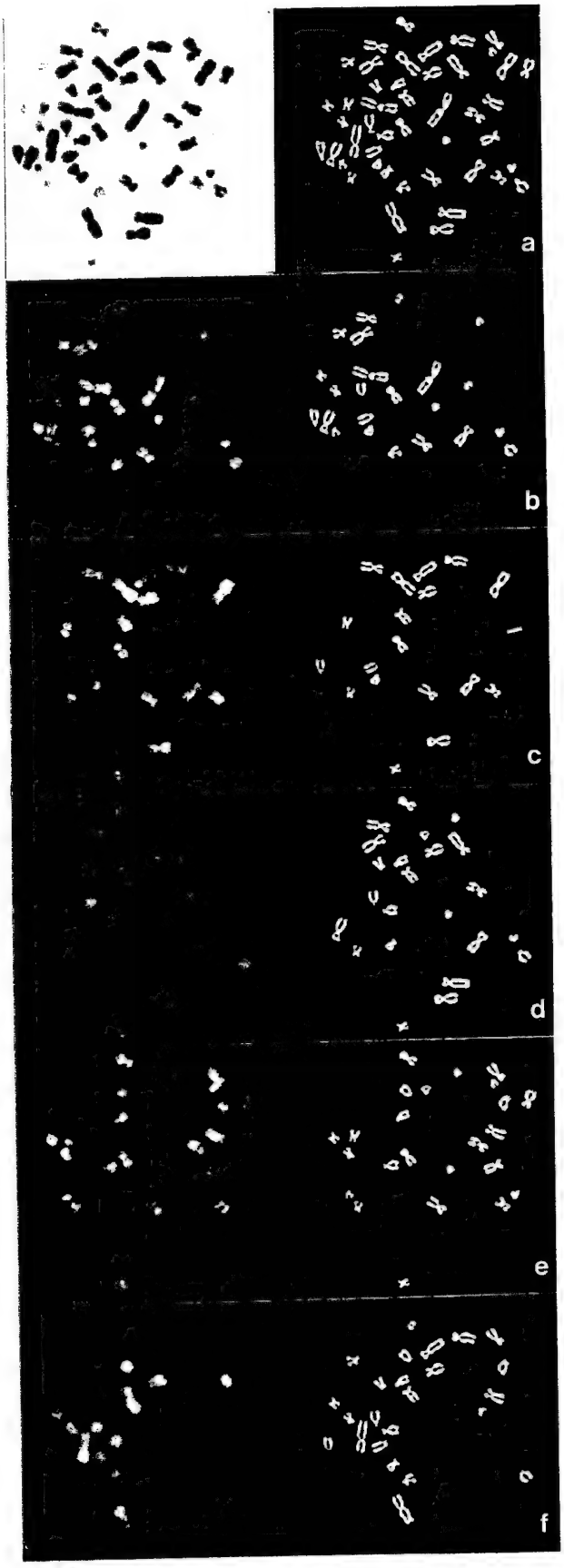
c) A schematic representation of the chromosomal rearrangements observed in the metaphase shown in b).

Fig. 8

a) DAPI banded normal male metaphase spread.

b) Same metaphase as in a) after hybridization with a probe cocktail containing 19 YAC-clones, 3 microdissected chromosome band specific probes, and 2 painting probes. For details see text.

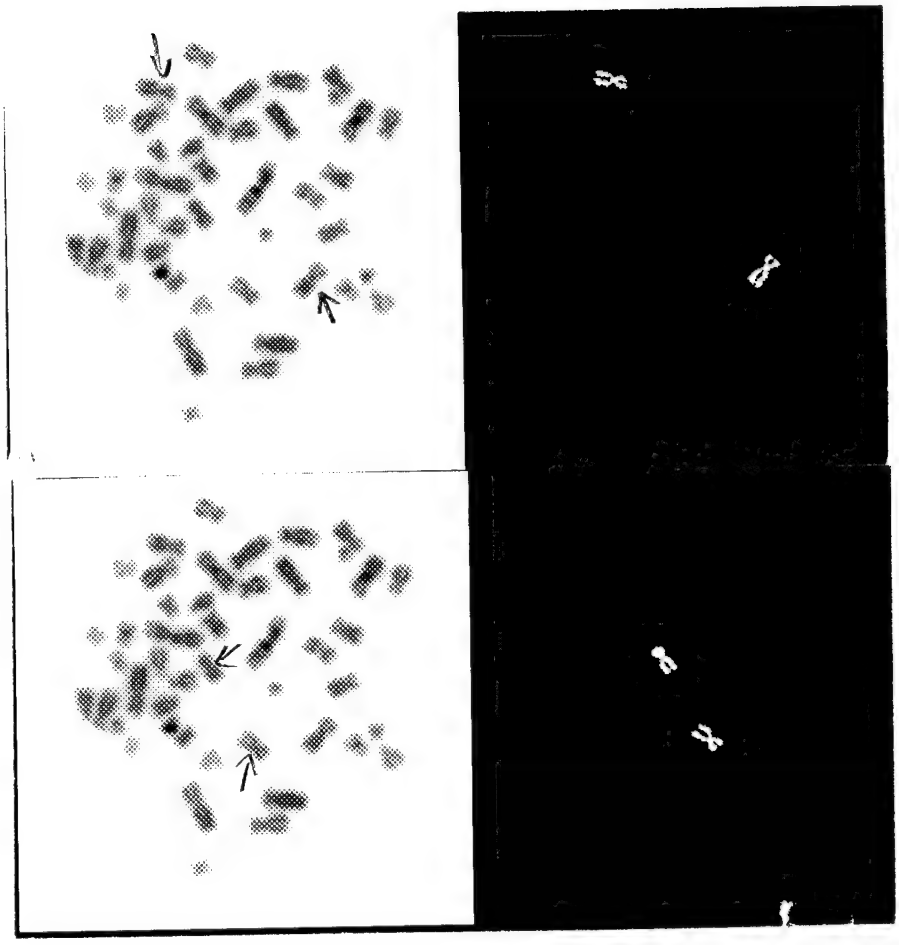
Figure 1



Cy3.5
Fluorescence Intensity Values
120
100
80
60
40
20
0
Threshold
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosomes

Figure 2

Figure 3



Chromosome 7

Chromosome 9

Figure 4

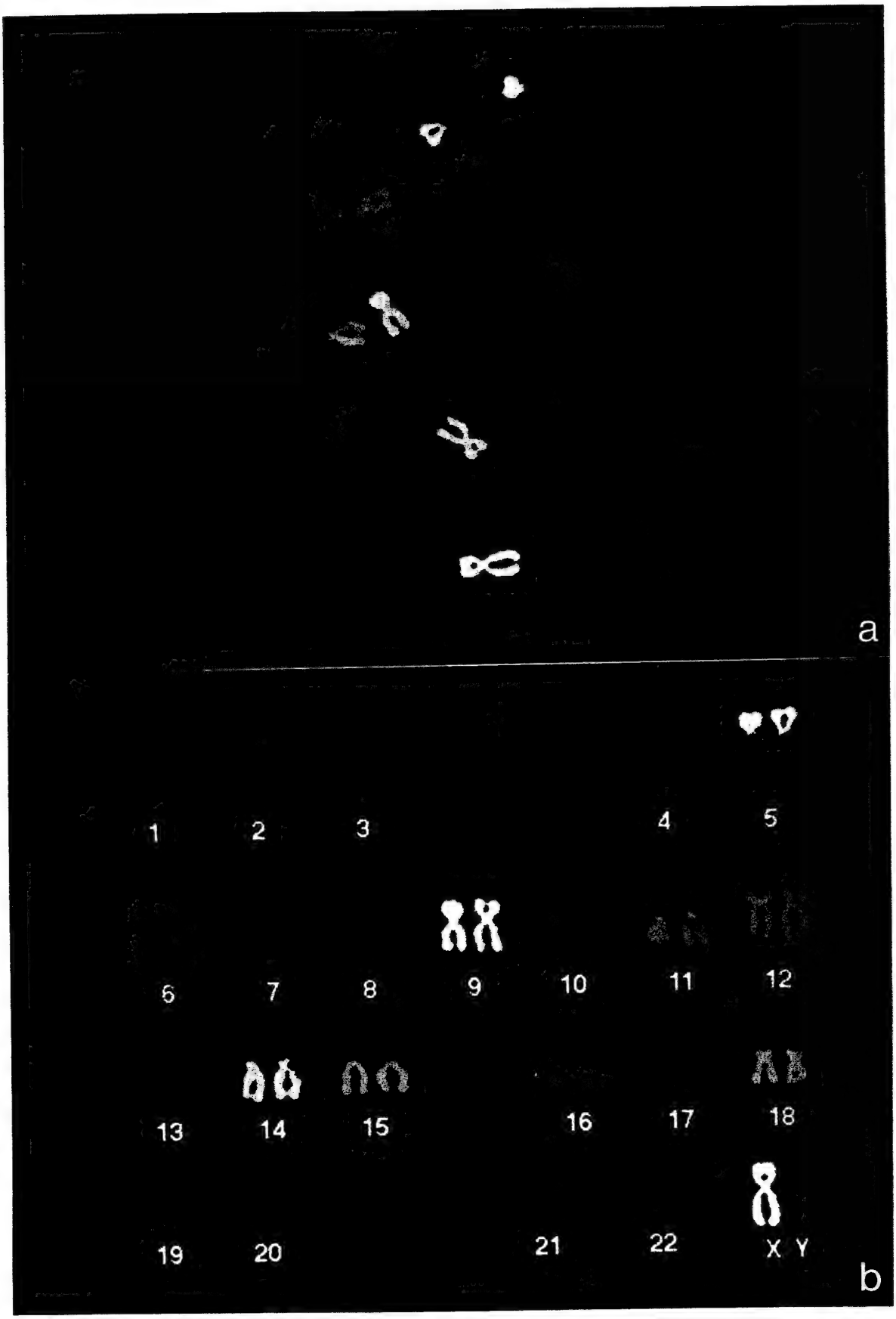
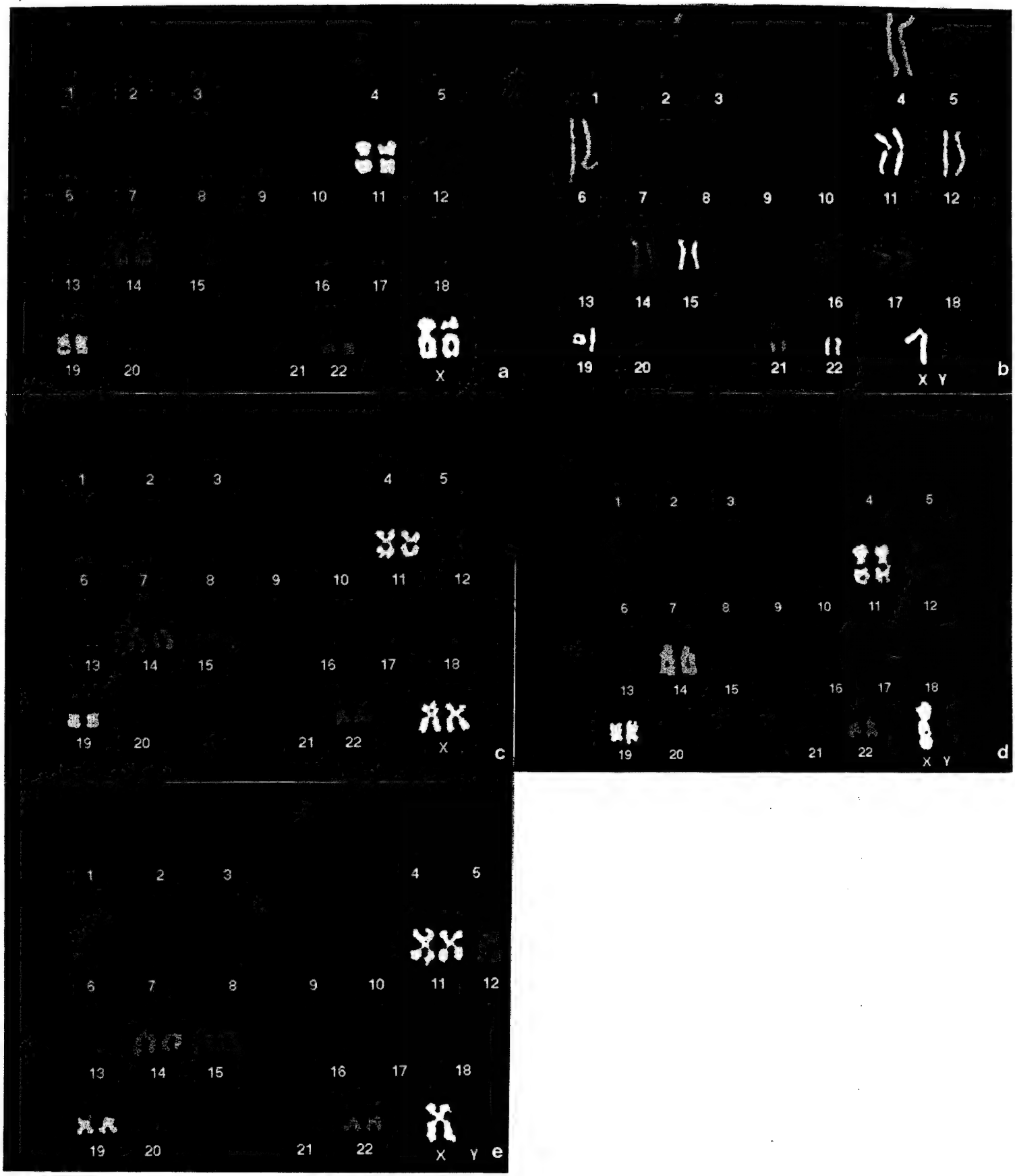


Figure 5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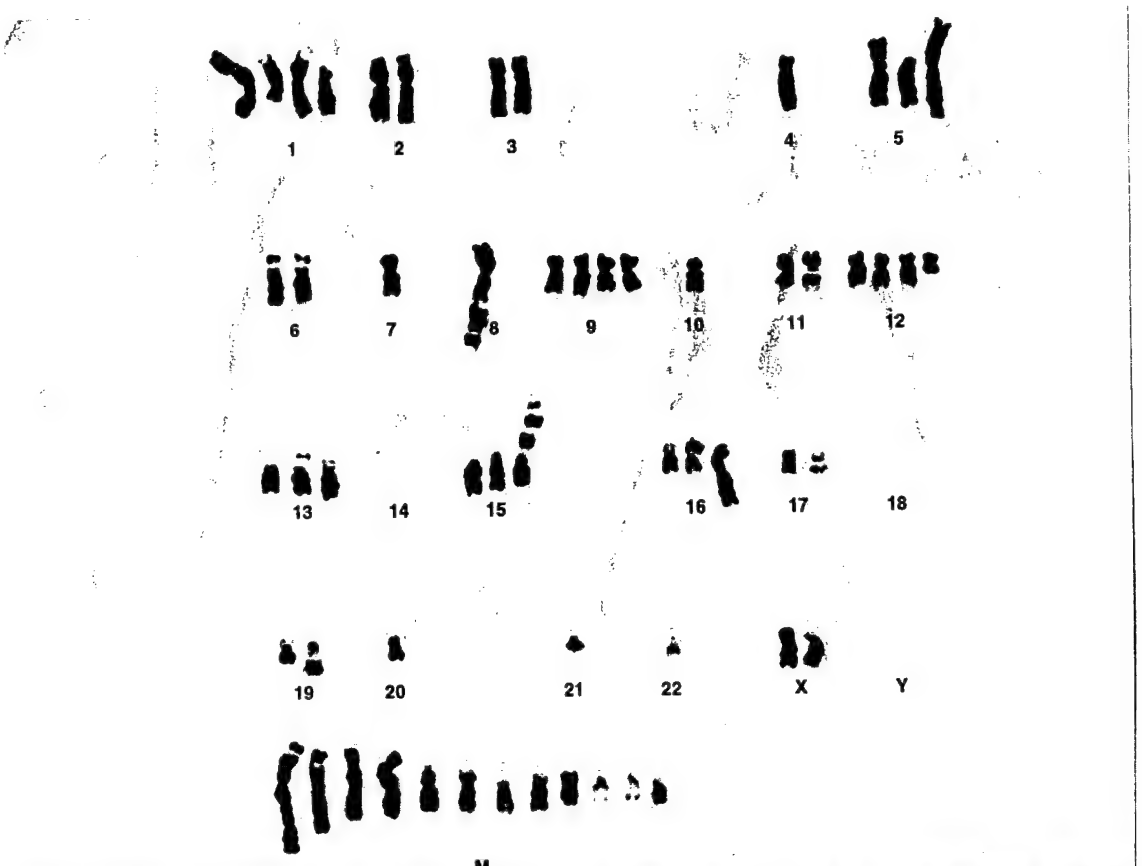
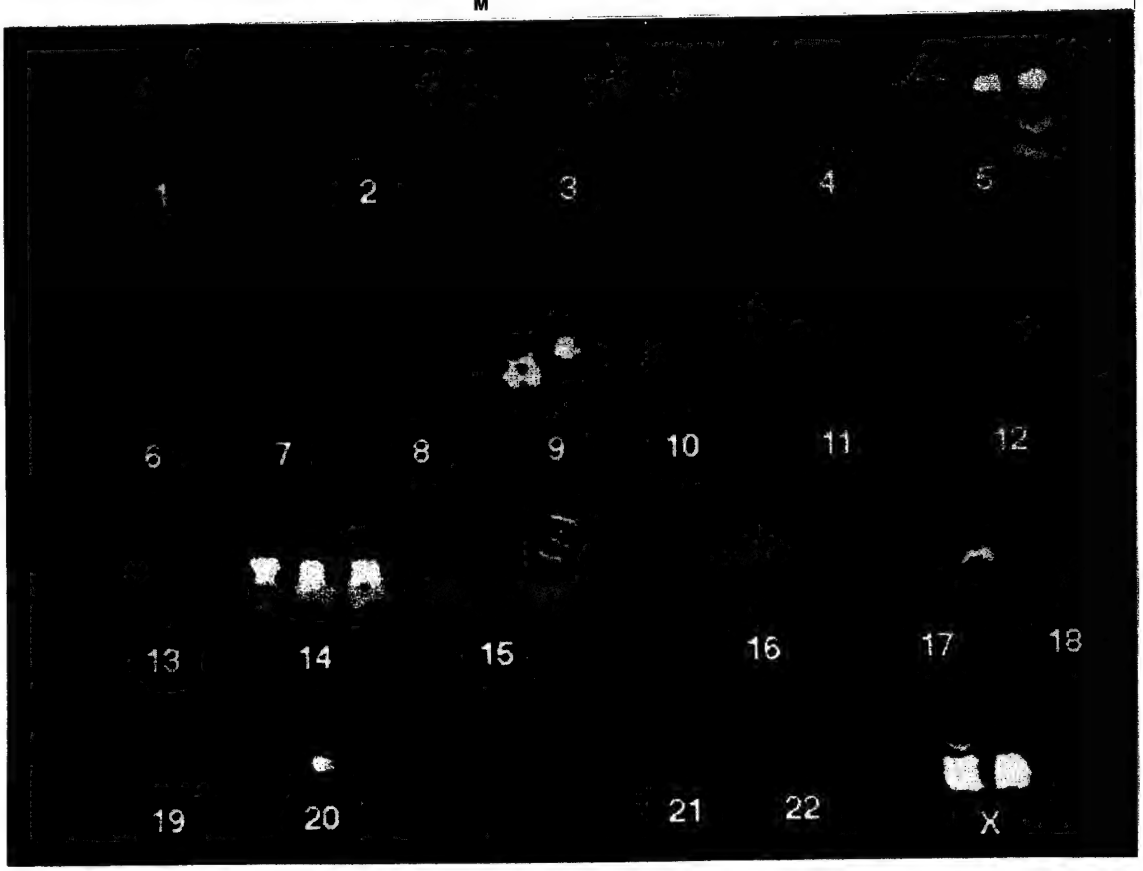
19 20 21 22 X Y e

Figure 6a and 6b





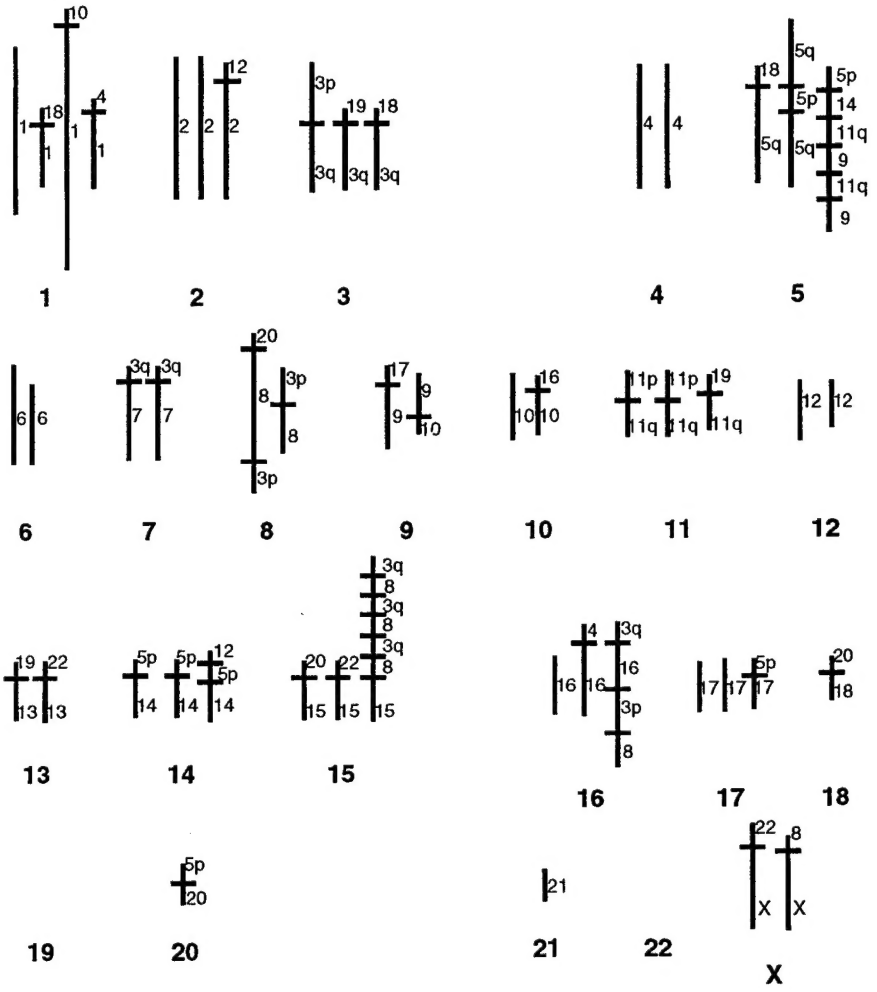
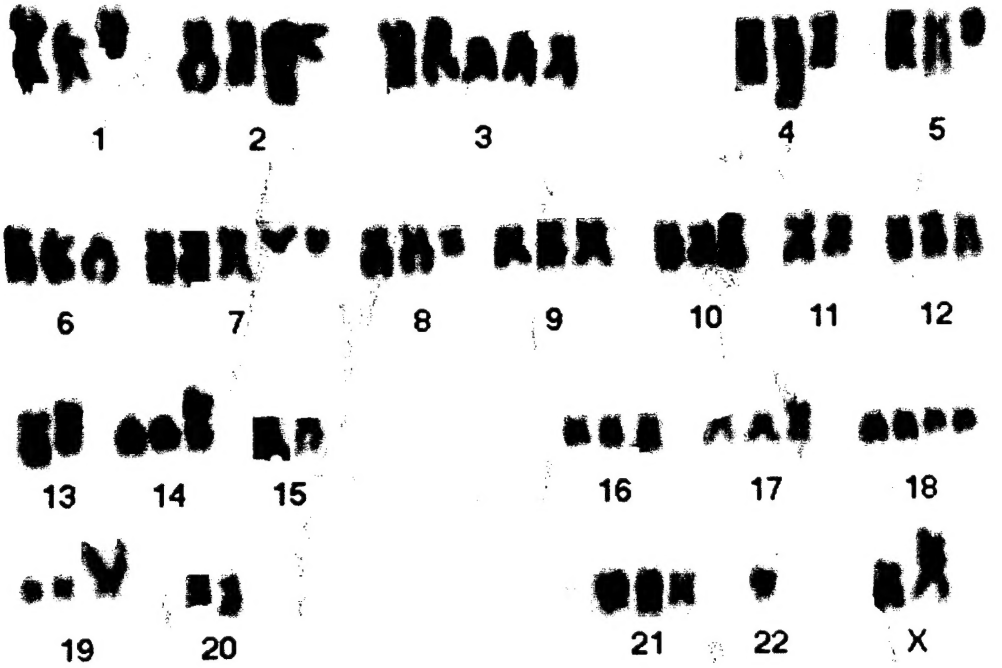
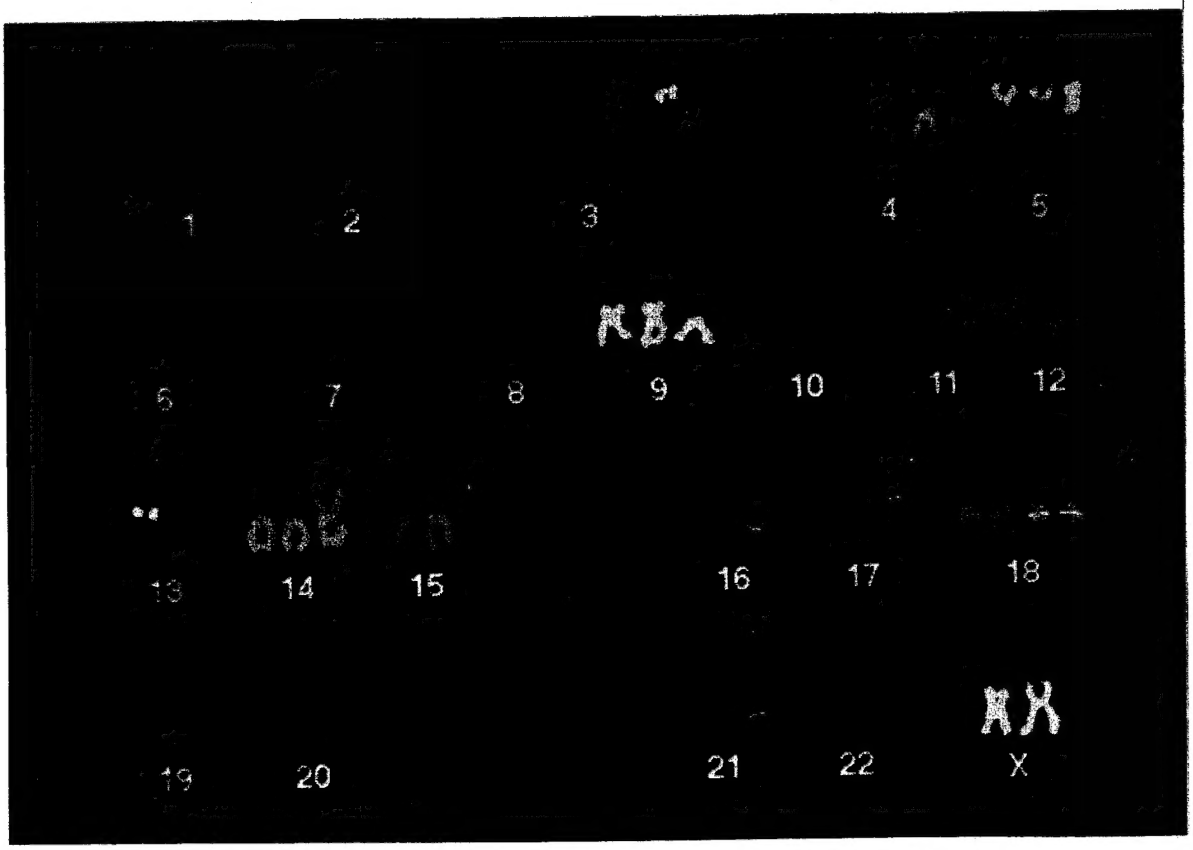


Fig. 6c

Figure 7a and 7b





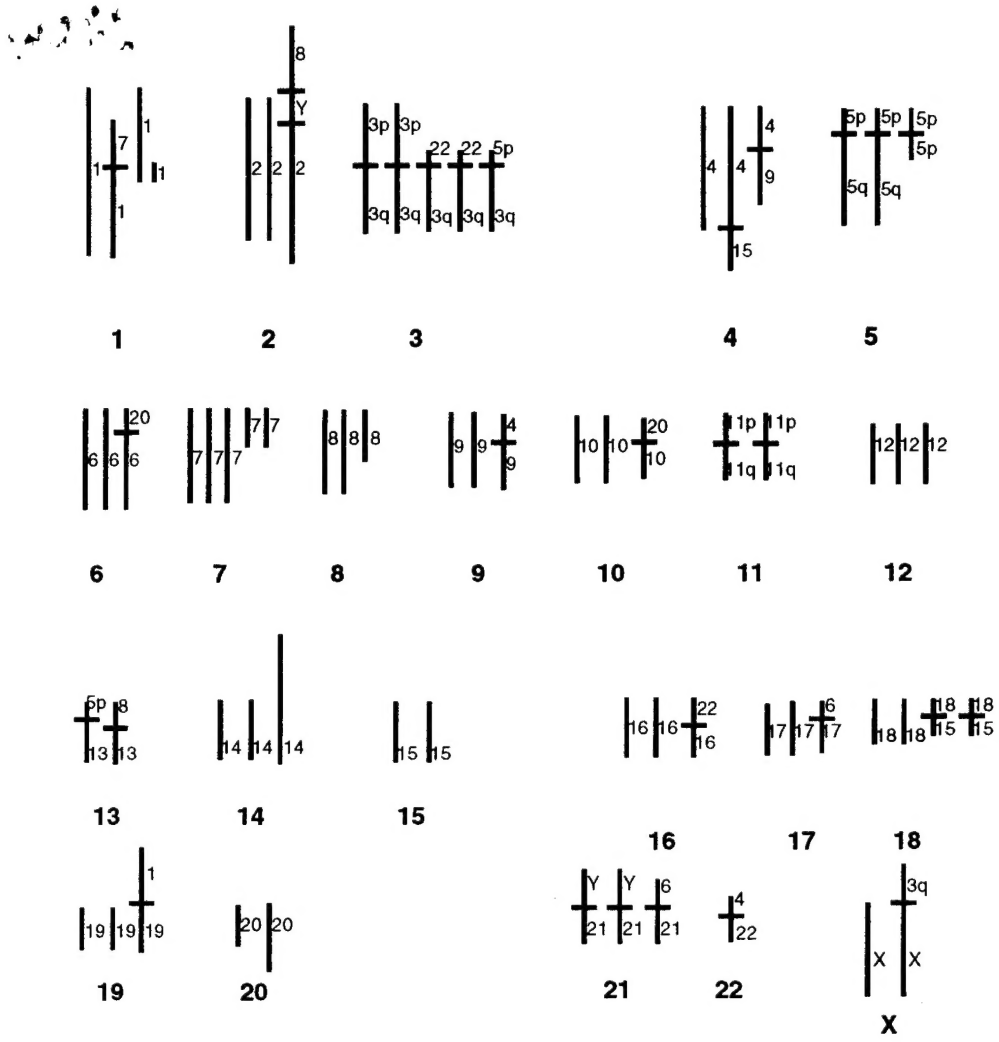


Fig. 7c

Figure 8a and 8b